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for

UNITED STATES LETTERS PATENT

on

CD40 ASSOCIATED PROTEINS

by

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and

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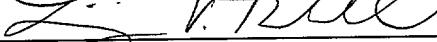
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CD40 ASSOCIATED PROTEINS  
BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to the fields of  
5 molecular biology and molecular medicine and more  
specifically to proteins involved in the regulation of  
immunological responses and cell growth.

BACKGROUND INFORMATION

Immunological responses can be broadly classified  
10 as humoral and cell-mediated. Humoral responses are  
mediated by antibody molecules present in plasma, lymph or  
tissue fluids and produced by plasma cells that  
differentiate from immature B cells. An antibody has both  
recognition and effector function that plays a key role in  
15 eliminating bacteria, neutralizing viruses and in  
triggering degranulation of mast cells and basophils.  
Cell-mediated immune responses are those mediated by  
antigen-specific T cells and various nonspecific cells of  
the immune system, such as natural killer cells, phagocytic  
20 cells and other white blood cells. Cell-mediated responses  
protect against infections due to intracellular bacteria,  
viruses or yeasts.

Despite the separate classifications of  
immunological responses, the humoral immune response is  
25 highly dependent for its function on a class of T cells  
known as T helper cells ( $T_h$  cells). Antigens that require  
 $T_h$  cells to elicit a humoral immune response are known as T-  
dependent antigens. During the humoral response to T-  
dependent antigens,  $T_h$  cells make direct contact with B  
30 cells, which results in B cell proliferation and  
immunoglobulin (Ig) class switching from the IgM class to  
IgG, IgA or IgE antibody classes. Various receptor-ligand  
interactions are involved in mediating contact between a  $T_h$   
cell and a B cell during the response to a T-dependent

antigen. In particular, CD40-CD40 ligand (CD40L) pairing is critical to achieving this cell-cell interaction.

CD40L and CD40 are both transmembrane glycoproteins that are homologous to tumor necrosis factor (TNF) and TNF receptors, (TNF-R), respectively. CD40 has a role in a variety of B cell functions, such as inducing Ig class-switching, acting as a cofactor with specific antigen and certain lymphokines for B cell mitogenesis, providing signals that prevent apoptotic cell death and triggering B cell adhesion to other cells. CD40L is not expressed on resting T<sub>H</sub> cells but can be induced after the cell contacts specific antigen.

The function of CD40/CD40L interactions in the immune response is revealed by hereditary abnormalities of CD40L, which cause an X-linked immunodeficiency syndrome. This syndrome is characterized by hyper-production of IgM, reduced levels of IgG, failure to produce IgA and IgE and absence of germinal centers in lymphoid tissues. The germinal centers are structures associated with T-dependent antibody responses. Nearly identical abnormalities are seen in transgenic mice that do not express CD40. These results indicate that the CD40/CD40L interaction provides a non-redundant pathway required for achieving T-dependent antibody responses *in vivo*.

It currently is unclear how CD40 transduces its CD40L-binding signal into a B cell. Since CD40 is present on the cell surface, its action likely is mediated by CD40 binding to one or more intracellular proteins, which ultimately effect signal transduction pathways that control Ig class-switching, cell proliferation and cell survival. However, the cytoplasmic domain of CD40 provides no clues as to how intracellular signalling is accomplished since the domain lacks homology to kinases or other enzymes known for mediating intracellular signalling.

The identification of intracellular proteins that can associate with CD40 and transduce the CD40L-binding signal into a cell would provide a means to manipulate various cellular functions, including, for example, humoral immune responses and apoptosis. Thus, a need exists to identify proteins that associate with CD40. The present invention satisfies this need and provides additional advantages as well.

SUMMARY OF THE INVENTION

10 The present invention provides substantially purified mammalian CD40-associated protein (CAP), which is related to the TRAF family of signal transducing proteins. The invention also provides nucleic acid molecules encoding CAPs, vectors containing the nucleic acid molecules and 15 host cells containing the vectors. For example, the invention provides a substantially purified human CAP-1 and a nucleic acid molecule encoding human CAP-1. The invention also provides antibodies that can specifically bind to a CAP.

20 The present invention also provides a screening assay useful for identifying agents that can effectively alter the association of a CAP with a second molecule that binds to the CAP in a cell. By altering the association of a CAP with a second molecule, an effective agent can 25 increase or decrease the level of apoptosis in a cell.

The invention further provides methods of identifying a CAP agonist or CAP antagonist that can increase or decrease, respectively, the level of expression of the CAP in a cell and thereby modulate a function of the 30 cell.

The invention also provides methods of detecting a CAP in a sample by detecting the presence of the CAP

protein or of a nucleic acid molecule encoding the CAP. Such methods can be used to diagnose a pathology that is characterized by an increased or decreased level of expression of a CAP in a cell.

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#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 lists the complete nucleotide sequence (SEQ ID NO:2) and the deduced amino acid sequence (SEQ ID NO:1) of CAP-1. Single letter amino acid symbols are used.

Figure 2 schematically shows the amino sequence 10 of CAP-1 and depicts the relative locations of the RING finger domains, zinc finger domains and the TRAF domain. Numbers indicate the amino acid position beginning from the N-terminus and ending at the C-terminus. Below the diagram are shown the regions of CAP-1 contributed by three 15 separate cDNA sequences.

Figure 3 schematically compares the structures of 20 TRAF-domain family of proteins CAP-1, TRAF1 and TRAF2. The positions of RING finger, zinc finger and TRAF domains are indicated. Numbers indicate the positions of amino acids beginning at the N-terminus.

Figure 4 provides a map of plasmid pBTM-116, which was used to produce LexA/CD40 fusion proteins. The plasmid contains a bacterial origin of replication (ori) and an ampicillin resistance gene (amp). The plasmid also 25 contains a yeast 2 micron ( $2 \mu\text{m}$ ) origin of replication and a gene that allows a yeast cell containing the plasmid to grow in the absence of tryptophan (TRP1). A LexA/CD40 fusion protein was produced by inserting a nucleotide sequence encoding the cytoplasmic domain of CD40 into the 30 EcoRI/BamHI sites located in the multiple cloning site. Cloning was performed so as to maintain the open reading

frame of LexA into the inserted sequence such that a LexA/CD40 fusion protein was produced.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel CD40-  
5 associated protein (CAP), designated CAP-1. As used herein, the term "CD40-associated protein" or "CAP" means a protein that binds specifically to CD40, which is a cell surface receptor involved in apoptosis. A CAP can be identified, for example, using the assays described in  
10 Examples I and II.

Although the term "CAP" is used generally, it should be recognized that a CAP identified using an assay described herein can be a portion of a protein, which is considered to be a candidate CAP. As used herein, the term 15 "candidate CAP" refers to a protein that corresponds to a peptide or polypeptide sequence that can bind CD40 but that consists of only a portion of the full length protein. For example, a CAP such as CAP-1 can be identified by obtaining cDNA sequences from a cDNA library, expressing the 20 polypeptides encoded by the cDNA sequences and detecting polypeptides that can bind CD40. Although such polypeptides are considered CAP's, it is well known that a cDNA sequence obtained from a cDNA library may not encode the full length protein. For example, a cDNA can encode a 25 polypeptide such as the CAP-1 cytoplasmic domain that is only a portion of the full length CAP-1 protein but, nevertheless, assumes an appropriate conformation so as to bind CD40. It is recognized, however, that in the full length protein, the polypeptide sequence can assume a 30 conformation that does not bind CD40 due, for example, to steric blocking of the CD40 binding site. Such a full length protein is an example of a candidate CAP. For convenience of discussion, the term "CAP" as used herein is intended to include a candidate CAP. Thus, a CAP can be a

protein or a polypeptide portion of a protein that binds CD40.

Since CD40 is critical for T cell-dependent antibody responses, the association of a CAP with CD40 can 5 affect the humoral immune response by modulating the level of production of IgG, IgA and IgE. CD40 is a ~50 kiloDalton (kDa) glycoprotein receptor that is expressed on the surface of B cells, some activated T cells, monocytes, follicular dendritic cells, basal epithelial cells as well 10 as some epithelial and non-epithelial carcinomas (Paulie et al., Immunotherapy 20:23-28 (1985); Paulie et al., J. Immunol. 142:590-595 (1989); Clark and Ledbetter, Proc. Natl. Acad. Sci., USA 83:4494-4498 (1986); Ledbetter et al., J. Immunol. 138:788-794 (1987); Young et al., Int. J. 15 Canc. 43:786-794 (1989)). The function of CD40 has been studied extensively in B cells, where it is believed to play an important role in regulation of cell proliferation, apoptosis, Ig class-switching, generation of memory B cells, modulation of cytokine gene expression and cell 20 adhesion (Clark and Ledbetter, Nature 367:425-428 (1994); Noelle et al., Today 13:431-433 (1992a); Lederman et al., Curr. Opin. Immunol. 5:439-444 (1993)). For example, CD40 can act as a cofactor in combination with anti-Immunoglobulin M (IgM) antibodies, anti-CD20 25 antibodies, phorbol myristic acetate or interleukin-4 (IL-4) to stimulate proliferation of resting primary B cells (Clark & Ledbetter, *supra*, 1986); Gordon et al., Eur. J. Immunol. 17:1535-1538 (1987)).

CD40 also provides signals that rescue germinal 30 center B cells from apoptotic cell death and that induce production of the apoptosis-blocking oncoprotein Bcl-2, suggesting a role for CD40 in the generation of long-lived memory B cells (Liu et al., Nature 342:929-931 (1989); Liu et al., Eur. J. Immunol. 21:1905-1910 (1991)). In 35 addition, CD40 synergizes with IL-4 to induce IgE synthesis

(Jabara et al., J. Exp. Med. 172:1861-1864 (1990); Gascan et al., J. Immunol. 147:8-13 (1991)) and cooperates with IL-10 or TGF- $\beta$  to stimulate IgA production (DeFrance et al., Amer. J. Exp. Med. 175:671-682 (1992)). Chimeric 5 proteins that contain the CD40 extracellular domain fused to the Fc portion of IgG, function as competitive antagonists of CD40 and can block B cell proliferation induced by activated T cells and inhibit Ig production stimulated by activated T cells and IL-4 or IL-5 (Noelle et 10 al., Proc. Natl. Acad. Sci., USA 89:6550-6554 (1992b)).

The ligand for CD40 (CD40L) is expressed on the surface of activated helper T cells, as well as mast cells and basophils as a type II transmembrane glycoprotein (Armitage et al., Nature 357:80-82 (1992); Hollenbaugh et 15 al., EMBO J. 11:4313-4321 (1992); Gauchet et al., Nature 365:340-343 (1993)). CD40L thus triggers CD40 action through a mechanism involving cell-cell contact. The importance of the CD40/CD40L interaction for the regulation 20 of T cell-dependent humoral immune responses was demonstrated by experiments in which antibodies to CD40L inhibited the generation of primary and secondary humoral immune responses (Foy et al., J. Exp. Med. 178:1567-1575 (1993)).

Alteration in CD40/CD40L interactions are 25 involved in human diseases. Mutations in the CD40L in humans causes an X-linked immunodeficiency syndrome characterized by excess production of IgM and by an absence of production of IgG, IgA and IgE due to a failure of B cells to undergo Ig class-switching (Allen et al., Science 30 259:990-993 (1993); DiSanto et al., Nature 361:541-543 (1993); Korthauer et al., Nature 361:539-543 (1993); Fuleihan et al., Proc. Natl. Acad. Sci., USA 90:2170-2173 (1993)). Thus, CD40L/CD40 interactions can be involved in Ig class-switching *in vivo*. Patients with hyper-IgM 35 syndrome have normal numbers of T cells and B cells and

intact cellular immune responses, but often develop lymph node hyperplasia and fail to form germinal centers (Clark and Ledbetter, *supra*, 1994). Likewise, transgenic mice with homozygous disruptions of their CD40 genes fail to 5 form germinal centers and do not produce IgG, IgA and IgE responses to T cell-dependent antigens, but can mount normal IgM and IgG responses to T cell-independent antigens (Kawabe et al., *Immunity* 1:167-178 (1994)). Antibodies to CD40L prevent collagen-induced arthritis in an animal model 10 (Durie et al., *Science* 261:1328-1330 (1993)), suggesting that inhibition of CD40-triggering could provide a means of therapeutically intervening in some autoimmune diseases. CD40L is expressed on mast cells and basophils, which play a key role in mediating atopic diseases. Since such cells 15 induce production of IgE synthesis by B cells co-stimulated with IL-4, the CD40/CD40L based interaction between B cells and either basophils or mast cells represents a critical point for controlling IgE-mediated atopic diseases (Gauchat et al., *Nature* 365:340-343 (1993)).

20 CD40 and CD40L are members of the tumor necrosis factor receptor (TNF-R) family and TNF family, respectively. The TNF-R family includes the p50/55 TNF-R1, p75/80 TNF-R2, p75 nerve growth factor (NGF) receptor, CD27, CD30 and Fas (Smith et al., *Cell* 76:959-962 (1994)). 25 The ligands for these receptors are trimeric protein complexes that presumably trigger their respective receptors by inducing receptor chain clustering. Molecular cloning of cDNA sequences for CD40 indicates that the extracellular domain of this receptor contains several 30 cysteine residues, whose positions are conserved among all members of this receptor family, and four copies of a repeated domain structure found in TNF-like receptors. Among the various members of the TNF-R family, CD40 is most 35 similar to p75 NGF-R (Stamenkovic et al., *EMBO J.* 8:1403-1410 (1989)).

The predicted cytoplasmic domain of CD40 contains 62 amino acids. The cytoplasmic domain bears no resemblance to kinases or other enzymes that might suggest a mechanism for signal transduction by this protein.

5 However, a region within the cytoplasmic domain of CD40 shares a limited degree of homology to a conserved domain found in the cytosolic tails of p75 NGF-R (22%), TNF-R1(31%) and Fas (41%). In TNF-R1 and Fas, this conserved domain is required for transducing a signal that

10 results in cell death, suggesting that this structure either is involved in receptor aggregation after ligand binding or represents a site that is required directly or indirectly for binding of signal transducing intracellular proteins to these receptors (Itoh et al., J. Biol. Chem. 15 268:10932-10937 (1993); Oehm et al., J. Biol. Chem. 267:10709-10715 (1992); Tartaglia et al., Cell 74:845-853 (1993)). Although CD40 and p75 NGF-R generate signals that suppress apoptosis, they also accelerate apoptosis when ectopically expressed in certain neural cell lines in the

20 absence of ligands (Rabizadeh et al., Science 261:345-348 (1993)). In such neural cells, CD40 and p75 NGF-R also can delay cell death relative to untransfected cells when triggered with appropriate ligands. Thus, under some circumstances, CD40 and NGF-R, like TNF-R and Fas, can 25 regulate signal transduction pathways leading to cell death.

Although the effects of CD40 on the regulation of apoptosis require the presence of the cytosolic domain, the biochemical mechanism of action remains unknown. In this 30 regard, antibody-mediated crosslinking of CD40 on the surface of B cells can induce turnover of phosphoinositides and phosphorylation of phosphatidyl-inositol-3'-kinase (PI3K), phospholipase-C- $\gamma$ , and several unknown intracellular proteins, and can increase the activities of 35 the protein tyrosine kinase Lyn and of PI3K (Uckun et al., J. Biol. Chem. 266:17478-17485 (1991); Gordon et al., J.

Immunol. 140:1425-1430 (1988); Ren et al., J. Exp. Med. 179:673-680 (1994)). In combination with IL-4, CD40 also can activate the transcription factor NF-AT (Choi et al., Immunity 1:179-187 (1994)). However, it is unclear how 5 these biochemical events are regulated by CD40 and what their relevance is to the biological effects of CD40 on B cells and other types of cells.

The identification herein of CAP-1 provides a means to manipulate the signal transduction pathways 10 controlled by CD40 and allows for the development of assays that are useful for identifying agents that effectively alter the association of CAP-1 with CD40. Such agents can be useful, for example, for effectively treating cancer in a subject or for treating an autoimmune disease, 15 immunodeficiency disease or neurodegenerative disease.

A CAP such as CAP-1 can be identified by detecting the association of the CAP with CD40. Such an association can be identified using an *in vivo* assay such as a yeast two hybrid assay (see Example I) or an *in vitro* 20 assay (see Example II). As used herein, the term "associate" or "association" means that a CAP and CD40 can bind to each other relatively specifically and, therefore, can form a bound complex. In particular, the association of a CAP, such as CAP-1, and CD40 is sufficiently specific 25 such that the bound complex can form *in vivo* in a cell or *in vitro* under suitable conditions (see Example II). Other methods for determining whether a protein can bind CD40 and, therefore, is a CAP are known in the art and include, for example, equilibrium dialysis.

30 In a normal cell, a steady state level of association of CAP-1 with CD40 can affect the normal level of apoptosis in that cell type. An increase or decrease in the steady state level of association of CAP-1 with CD40 in a cell can result in an increased or decreased level of

apoptosis in the cell, which can result in a pathology in a subject.

The normal association of CAP-1 with CD40 in a cell can be altered due, for example, to the expression in 5 the cell of a variant CAP-1 or a variant CD40, either of which can compete for binding with CAP-1 that normally binds to CD40 in the cell. In this case, the association of CAP-1 with CD40 can be decreased in a cell. The term "variant" is used generally herein to mean a protein such 10 as CAP-1 or CD40 that is different from the protein normally found in a particular cell type. The normal association of CAP-1 with CD40 in a cell also can be increased or decreased due, for example, to contact of the cell with an agent such as a drug that can effectively 15 alter the association of CAP-1 with CD40 in a cell.

CAP-1 was identified using the yeast two hybrid system (Fields and Song, Nature 340:245-246 (1989); Vojtek et al., Cell 74:205-214 (1993); Durfee et al., Genes Devel. 7:555-569 (1993), each of which is incorporated herein by 20 reference). An *in vivo* transcription activation assay such as the yeast two hybrid system is particularly useful for identifying and manipulating the association of proteins. The results observed using such an assay likely mirror the interactions that naturally occur in a cell. Thus, the 25 results obtained in such an *in vivo* assay can be predictive of results that can occur in a cell in a subject such as a human subject.

A transcription activation assay such as the yeast two hybrid system is based on the modular nature of 30 transcription factors, which consist of functionally separable DNA-binding and trans-activation domains. When expressed as separate proteins, these two domains fail to mediate gene transcription. However, transcription activation activity can be restored if the DNA-binding

domain and the trans-activation domain are bridged together due, for example, to the association of two proteins. The DNA-binding domain and trans-activation domain can be bridged, for example, by expressing the DNA-binding domain 5 and trans-activation domain as fusion proteins (hybrids), provided that the proteins that are fused to the domains can associate with each other. The non-covalent bridging of the two hybrids brings the DNA-binding and trans-activation domains together and creates a transcriptionally 10 competent complex. The association of the proteins is determined by observing transcriptional activation of a reporter gene (see Example I).

The yeast two hybrid system exemplified herein uses various strains of *S. cerevisiae* as host cells for 15 vectors that express the hybrid proteins. A transcription activation assay also can be performed using, for example, mammalian cells. However, the yeast two hybrid system is particularly useful due to the ease of working with yeast and the speed with which the assay can be performed. For 20 example, yeast host cells containing a lacZ reporter gene linked to a LexA operator sequence were used to demonstrate that CAP-1 can interact with CD40 (Example I). The DNA-binding domain consisted of the LexA DNA-binding domain, which binds the LexA promoter, fused to a portion of CD40 25 and the trans-activation domain consisted of a GAL4 trans-activation domain fused to cDNA sequences, some of which encoded a CAP. When the LexA domain was non-covalently bridged to a trans-activation domain fused to a CAP, the association of CD40 and CAP-1 was identified by activated 30 transcription of the reporter gene.

A CAP also can be identified using an *in vitro* assay such as an assay utilizing, for example, a glutathione-S-transferase (GST) fusion protein as described in Example II. Such an *in vitro* assay provides a simple, 35 rapid and inexpensive method for identifying and isolating

a CAP. Such an *in vitro* assay is particularly useful for confirming results obtained *in vivo* and can be used to characterize specific binding domains of a CAP (see Example II). For example, a GST/CD40 fusion protein can be 5 expressed and can be purified by binding to an affinity matrix containing immobilized glutathione. If desired, a sample that can contain a CAP or an active fragment of a CAP can be passed over an affinity column containing bound GST/CD40 and a CAP that binds to CD40 can be obtained. 10 GST/CD40 also can be used to screen a cDNA expression library, wherein binding of the CD40 fusion protein to a clone indicates that the clone contains a cDNA encoding a CAP. In addition to it's use in detecting and purifying a CAP, the *in vitro* assay provides a useful method to screen 15 for a small molecule inhibitor of a particular CAP-CD40 interaction.

Using these *in vitro* and *in vivo* assays, CAP-1 was demonstrated to associate with CD40. CAP-1 contains a C-terminal domain with strong amino acid sequence homology 20 to a conserved region that is present in two putative signal transducing proteins that bind to the cytosolic domain of p75/80 TNF-R2. These proteins have been designated TNF-R2 associated factors-1 and -2 (TRAF1 and TRAF2) (Rothe et al., Cell 78:681-692 (1994)). The TRAF 25 domain in CAP-1 can mediate the interactions of CAP-1 with CD40 and allows for homodimerization of CAP-1 proteins. Like the TRAF2 protein, CAP-1 also contains a RING finger motif and zinc finger-like domains, similar to those found in several regulatory proteins that can interact with DNA 30 or RNA (see Figure 3 and Example III). CAP-1 thus represents a novel member of a family of proteins that can interact with the cytoplasmic domains present in the TNF-R family proteins and can be involved in signal transduction in a cell.

The present invention provides a substantially purified mammalian CAP or an active fragment thereof, which can bind CD40. In addition, the invention provides human CAP-1 having substantially the amino acid sequence shown in 5 Figure 1 (SEQ ID NO: 1). The amino acid sequence of human CAP-1 was derived from the nucleotide sequence shown in Figure 1 (SEQ ID NO: 2).

As used herein, the term "substantially the amino acid sequence" means the disclosed amino acid sequence for 10 human CAP-1 (SEQ ID NO: 1) as well as amino acid sequences that are similar to SEQ ID NO: 1, but have one or more amino acid additions, deletions or substitutions that do not substantially alter the ability of the encoded protein to function like CAP-1 and bind to CD40 or to another 15 protein such as a member of the TRAF family of proteins. As used herein, the term "substantially purified" means a protein that is in a form that is relatively free from contaminating lipids, proteins, nucleic acids or other cellular material normally associated with a protein in a 20 cell. A substantially purified human CAP-1 protein can be obtained, for example, using well known biochemical methods of purification or by expressing a recombinant nucleic acid molecule encoding CAP-1 such as the nucleic acid molecule shown as SEQ ID NO: 2. In addition, an amino acid sequence 25 consisting of at least a portion of the amino acid sequence of SEQ ID NO: 1 can be chemically synthesized or can be produced by expressing a portion of the nucleotide sequence shown as SEQ ID NO: 2.

As used herein, the terms "protein" or 30 "polypeptide" are used in the broadest sense to mean a sequence of amino acids that can be encoded by a cellular gene or by a recombinant nucleic acid sequence or can be chemically synthesized. In some cases, the term "polypeptide" is used in referring to a portion of an amino 35 acid sequence encoding a full length protein. An active

fragment of CAP-1 as defined below can be an example of such a polypeptide. A protein can be a complete, full length gene product, which can be a core protein having no amino acid modifications or can be a post-translationally modified form of a protein such as a phosphoprotein, glycoprotein, proteoglycan, lipoprotein or nucleoprotein.

A CAP such as a CAP-1, can be characterized by its ability to associate with CD40. As used herein, the term "CD40" means the full length CD40 protein or a portion of the full length CD40 protein such as the CD40 (positions 220-271) or CD40 (positions 225-269) portions of CD40, either of which can associate with CAP-1 (see Example II). It should be recognized that the ability of a CAP to associate with CD40 can be due to a portion of the full length CAP as is demonstrated herein using a portion of CAP-1.

As used herein, the term "active fragment" means a CAP that is a portion of a full length protein, provided that the portion has an activity that is characteristic of the corresponding full length protein. For example, an active fragment of CAP-1, such as the cytoplasmic domain can have an activity such as the ability, for example, to bind CD40 or to mediate signal transduction after binding to CD40 or to elicit an immune response. The characteristic of an active fragment of a CAP to elicit an immune response is useful for obtaining an anti-CAP antibody. Thus, the invention also provides active fragments of a CAP, which can be identified using the assays described below.

The full length CAP-1 protein contains 543 amino acids and has an estimated molecular mass of 62 kDa. The cytosolic region of CAP-1 binds to a 45 amino acid cytosolic domain of CD40 (positions 225 to 269) that is weakly homologous (22 to 41%) to domains found in the

cytosolic portions of TNF-R1, Fas, and p75 NGF-R. This cytosolic domain is required for TNF-R1-mediated and Fas-mediated cytotoxicity (Itoh et al., Cell 66:233-243 (1993); Tartaglia et al., Cell 74:845-853 (1993)).

5        The cytoplasmic TRAF domain of CAP-1 can bind to CD40 and can form dimers as disclosed herein (see Examples I and II). The TRAF1 and TRAF2 proteins can form homotypic and heterotypic dimers (Rothe et al., *supra*, 1994), suggesting their TRAF domains mediate dimer formation in a  
10 manner similar to the TRAF domain of CAP-1. In view of the structural similarities between the TRAF domains of TRAF1, TRAF2 and CAP-1, it is likely that all three proteins can form dimers with each other in a cell. Heterodimer formation between TRAF family members can provide an  
15 additional degree of specificity for regulating signal transduction in different cell types.

      CAP-1 contains several sequences that are homologous to domains commonly found in DNA binding proteins. These sites include a RING finger domain (see  
20 Freemont, Ann. New York Acad. Sci. 174-172 (1993); Schwabe and Klug, Struct. Biol. 1:345-349 (1994); Barlow et al., J. Mol. Biol. 237:201-211 (1994)) and three zinc finger domains. TRAF2 is similar to CAP-1 in having a single RING finger domain and in having multiple zinc finger domains,  
25 while TRAF1 lacks these domains (see Figures 2 and 3). The presence of DNA binding motifs in CAP-1 and TRAF2 indicates these proteins can have a role in the regulation of gene transcription.

      The present invention provides a reagent that  
30 specifically binds to a CAP. As used herein the term "reagent" means a chemical or biological molecule that can specifically bind to a CAP. Examples of a reagent include CD40, an anti-CAP antibody or a protein containing a TRAF domain. As used herein, the term "antibody" is used in its

broadest sense to include polyclonal and monoclonal antibodies, as well as polypeptide fragments of antibodies that retain a specific binding activity for a specific antigen of at least about  $1 \times 10^5 \text{ M}^{-1}$ . One skilled in the art would know that anti-CAP antibody fragments such as Fab,  $\text{F}(\text{ab}')_2$ , Fv and Fd fragments can retain specific binding activity for a CAP and, thus, are included within the definition of an antibody. In addition, the term "antibody" as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies and fragments of antibodies that retain binding activity. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains as described by Huse et al., Science 246:1275-1281 (1989), which is incorporated herein by reference.

An anti-CAP antibody can be prepared using well known methods as described, for example, by Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press, 1988), which is incorporated herein by reference. For example, CAP-1 protein or a portion of the CAP-1 protein can be used as an immunogen. Such an immunogen can be prepared from natural sources or produced recombinantly or, in the case of a portion of the protein, can be chemically synthesized. Non-immunogenic peptides of a CAP protein can be made immunogenic by coupling to a carrier molecule such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) as described, for example, by Harlow and Lane (*supra*, 1988). In addition, a CAP fusion protein can be expressed as described in Example II and can be purified and used as an immunogen to obtain anti-CAP-1 antibodies.

Polyclonal antibodies can be raised, for example, in rabbits. In addition, monoclonal antibodies can be obtained using well known methods (see, for example, Reed et al., Anal. Biochem. 205:70-76 (1992), which is 5 incorporated herein by reference; see, also, Harlow and Lane, *supra*, 1988). For example, spleen cells from a CAP-1 immunized mouse can be fused to an appropriate myeloma cell line such as SP2/0 or P3x653.Ag8 myeloma cells to produce hybridoma cells. Cloned hybridoma cell lines can be 10 screened using a labelled CAP-1 immunogen to identify clones that secrete monoclonal antibodies. Hybridomas that express antibodies having a desirable specificity and affinity can be isolated and utilized as a continuous source of antibodies. One skilled in the art would know 15 that a dependable source of monoclonal antibodies is desirable, for example, for preparing diagnostic kits as described below.

The invention also provides a substantially purified nucleic acid molecule encoding a CAP. For 20 example, the invention provides a substantially purified nucleic acid molecule having substantially the nucleotide sequence encoding human CAP-1 shown in Figure 1 (SEQ ID NO: 2). Although the disclosed cDNA sequence lacks a 25 polyadenylation site, it contains the complete open reading frame encoding human CAP-1.

As used herein, the term "substantially purified" means a nucleic acid molecule that is in a form that is relatively free from contaminating lipids, proteins, nucleic acids or other cellular material normally 30 associated with a nucleic acid molecule in a cell. A substantially purified nucleic acid molecule can be obtained, for example, by recombinant DNA methods as described herein (see, also, Sambrook et al., Molecular Cloning: A laboratory manual (Cold Spring Harbor Laboratory

Press 1989), which is incorporated herein by reference) or can be chemically synthesized.

As used herein, the term "substantially the nucleotide sequence" means the disclosed nucleotide sequence for human CAP-1 (SEQ ID NO: 2), as well as a similar sequence that contains, for example, different nucleotides than shown in SEQ ID NO: 2, but that, as a result of the degeneracy of the genetic code, encodes the same amino acid sequence as shown in SEQ ID NO: 1. In 10 addition, a nucleic acid molecule of the invention can encode a portion of CAP-1 or can encode a CAP-1 having substantially the amino acid sequence shown in Figure 1 (SEQ ID NO: 1).

The invention also provides a nucleotide sequence 15 that can hybridize to a portion of a nucleic acid molecule encoding a mammalian CAP under relatively stringent hybridization conditions. Such a nucleotide sequence should be at least ten nucleotides in length and can be prepared, for example, by restriction endonuclease 20 digestion of a cloned nucleic acid molecule encoding a CAP, such as CAP-1, or by PCR amplification of a portion of the nucleic acid molecule shown in Figure 1 (SEQ ID NO: 2), or by chemical synthesis. Relatively stringent hybridization conditions can be determined empirically or can be 25 estimated based, for example, on the relative GC:AT content of the hybridizing nucleotide sequence and the target sequence, the length of the hybridizing nucleotide sequence and the number, if any, of mismatches between the hybridizing nucleotide sequence and the target sequence. 30 If desired, a hybridizing nucleotide sequence can be detectably labelled and used as a probe or can be used as a primer for PCR. Methods for detectably labelling a nucleotide sequence are well known in the art (see, for example, Sambrook et al., *supra*, 1989; see, also, Ausubel 35 et al., Current Protocols in Molecular Biology vol. 2,

chapter 10 (Greene Publ., NY 1989), which is incorporated herein by reference).

A CAP also can be a mutant CAP, which is a CAP that contains at least one or a few amino acid additions, 5 deletions or substitutions that result in the loss of an activity of the CAP but that do not significantly alter the ability of the mutant CAP to bind CD40 or a protein having a TRAF domain. For example, a mutant CAP can lose the ability to mediate signal transduction following binding to 10 CD40. Such a mutant CAP can modulate an immune response, for example, by binding to CD40, thereby preventing binding of a wild type CAP to CD40 in a cell. As used herein, the term "modulate" means increase or decrease. A mutant CAP can be obtained, for example, by site directed mutagenesis 15 or codon based mutagenesis of a nucleic acid molecule encoding a CAP and screening the mutagenized nucleic acid molecule to determine whether it encodes a mutant CAP, which can bind CD40 but lacks a CAP activity (see, for example, U.S. Patent No. 5,223,409 and U.S. Patent No. 20 5,264,563, each of which is incorporated herein by reference).

The determination that a CAP such as CAP-1 can bind CD40 or a protein having a TRAF domain provides a means to identify agents that can effectively alter the 25 association, for example, of CAP-1 with CD40. Thus, the present invention provides a screening assay useful for identifying an effective agent, which can alter the association of CAP-1 with CD40. In addition, since CD40 is involved in apoptosis, such effective agents can be useful 30 for modulating the level of apoptosis in a subject having a pathology characterized, for example, by an increased or decreased level of cell growth or cell proliferation.

The methods disclosed herein are useful for identifying an agent that alters the association of a CAP

with a second molecule in a cell. As used herein, the term "second molecule" means a cellular molecule that can bind to a CAP. Such a second molecule can include, for example, CD40 or a TRAF domain-containing protein such as CAP-1,  
5 TRAF1 or TRAF2. A second molecule also can be a nucleotide sequence such as a DNA or RNA sequence that can bind to a CAP.

A nucleotide sequence that can bind to a CAP can be detected by using methods well known in the art (see for  
10 example, El-Deiry et al., Nat. Gen. 1:45 (1992), which is incorporated herein by reference). Genomic DNA can be processed to produce uniform-sized fragments using, for example, sonication and the fragments can be screened for the ability to bind to a CAP such as CAP-1. Genomic DNA  
15 sequences that bind to a CAP can be isolated using an anti-CAP antibody followed by Protein A chromatography. Such isolated DNA sequences can be amplified by PCR, which can be facilitated by ligating the original genomic DNA fragments to "catch linkers" (El-Deiry et al., *supra*, 1992)  
20 suitable for annealing to PCR primers.

Alternatively, instead of screening genomic DNA fragments to identify a CAP binding nucleotide sequence, random oligonucleotides consisting of about twelve to about sixteen nucleotides, and including "catch linkers," can be  
25 screened for sequences that can bind a CAP such as CAP-1, for example, by immobilizing the CAP protein to a filter, then incubating the filter with the oligonucleotides under conditions that allow the CAP to bind relatively specifically to a CAP binding sequence. Unbound  
30 oligonucleotides can be washed from the filter, then specifically bound sequences can be eluted and amplified by PCR. Following three or more cycles of binding, elution and amplification, a consensus CAP binding sequence can be obtained. If desired, the consensus CAP binding sequence

can be used to screen a genomic DNA library to obtain genomic DNA sequences containing the CAP binding sequence.

As used herein, the term "agent" means a chemical or biological molecule such as a simple or complex 5 organic molecule, a peptide, a peptido-mimetic, a protein, a carbohydrate or an oligonucleotide that has the potential for altering the association of a CAP with a second molecule or altering an activity of a CAP. In addition, the term "effective agent" is used herein to mean an agent 10 that can, in fact, alter the association of a CAP with a second molecule or alter the activity of a CAP. For example, an effective agent can be one that alters the association of CAP-1 with CD40. An effective agent that alters the association of a CAP with a second molecule can 15 interfere with the ability of a CAP to associate with the second molecule or can cause the dissociation of a bound second molecule-CAP complex, either of which can modulate the function of a cell. Thus, an effective agent can be useful as a medicament to treat pathology characterized, in 20 part, by an abnormal cell function.

As used herein, the term "alter the association" means that the association of a CAP with a second molecule either is increased or is decreased due to the presence of an effective agent. As a result of an altered association 25 of a CAP with a second molecule in a cell, a function of the cell can be modulated. As used herein, modulating "the function of a cell" refers to any cellular function or cell state that can be effected by a CAP in a cell including, for example, Ig class-switching, cell proliferation and 30 apoptosis. As used herein, the term "alter the activity" means that the effective agent can increase or decrease an activity such as the signal transducing activity of a CAP in a cell. As a result of the altered activity of a CAP, the function of a cell can be modulated.

An effective agent can be useful for blocking the Ig class-switching signal that results due to the binding of CD40L of a T<sub>H</sub> cell with CD40 of a B cell. The ability to block Ig class-switching can reduce the level of IgE

5 production by an individual, thus, providing a means to treat atopic diseases mediated by IgE class immunoglobulin. Such atopic diseases include, for example, asthma, hay-fever, chronic sinusitis, skin rashes such as urticaria, or allergies such as those resulting from contact with a food,

10 plant, plant pollen or animal dander. Similarly, autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), autoimmune hemolytic anemia or glomerulonephritis in which production of IgA and IgG class immunoglobulins is involved, can be treated using

15 an effective agent that modulates Ig class-switching.

An effective agent also can be useful to decrease the proliferation of, for example, a cancer cell, which is characterized by having an increased rate of cell division or a decreased rate of cell death as compared to its normal

20 cell counterpart. Thus, an effective agent can be used as a medicament to treat cancer by increasing the level of apoptosis in a cancer cell that expresses CD40 or a protein having a TRAF domain. An effective agent also can be useful for increasing the level of apoptosis in a cell such

25 as a B cell in a subject having an autoimmune disease such as a RA or SLE, which is characterized by an increased level of production of specific immunoglobulins that react with host tissues. Such diseases are characterized, in part, by a reduced level of apoptosis, which is a pathway

30 ordinarily used to effectively eliminate autoreactive antibody producing B cells from an individual.

An effective agent also can be useful for decreasing the level of apoptosis or increasing Ig class-switching in B cells from a patient having an

35 immunodeficiency, including a hereditary disorder such as

X-linked hyper-IgM syndrome or an acquired disorder such as AIDS. Thus, an effective agent can be useful as a medicament for modulating apoptosis in a subject having a pathology characterized by decreased antibody production, 5 decreased cell proliferation or increased apoptosis. In addition, an effective agent can be used to decrease the level of apoptosis and, therefore, increase the survival time of a cell such as a hybridoma cell in culture. The use of an effective agent to prolong the survival of a cell 10 *in vitro* can significantly improve bioproduction yields in industrial tissue culture applications.

A mutant CAP that lacks an activity such as signal transducing activity but retains the ability to associate with a second molecule is an example of an 15 effective agent, since the expression of a mutant CAP in a cell can alter the association of a CAP with a second molecule. Thus, it should be recognized that a mutant CAP can be an effective agent. In addition, an active fragment 20 of a CAP can be an effective agent that alters the normal association of a CAP and a second molecule in a cell. Such active fragments, which can be peptides as small as about five amino acids, can be identified, for example, by screening a peptide library to identify peptides that can bind CD40 or a TRAF domain- containing protein (see, for 25 example, U.S. Patent No. 5,223,409).

Similarly, a peptide or polypeptide portion of a second molecule can be an effective agent. For example, the C-terminal amino acid sequence of CD40 is a second molecule that can associate with CAP-1 (see Example II). 30 A peptide derived from the C-terminal portion of CD40 can be useful, for example, for decreasing the association of wild type CD40 with CAP-1 in a cell by competing for binding to wild type CAP-1. In a similar way, a peptido-mimetic can be useful as an effective agent. Such a 35 peptido-mimetic can include, for example, a peptoid, which

is peptide-like sequence containing N-substituted glycines, or an oligocarbamate. A peptido-mimetic can be particularly useful as an effective agent due, for example, to having an increased stability to enzymatic degradation

5 *in vivo*.

As disclosed herein, a screening assay can be performed *in vivo* using the yeast two hybrid system or can be performed *in vitro*. The yeast two hybrid system, for example, can be used to screen a panel of agents to

10 identify effective agents that alter the association of a second molecule with CAP-1. As described above, a second molecule can be CAP-1 itself, a TRAF domain-containing protein, such as TRAF1, TRAF2 or CAP-1 or a nucleotide sequence that specifically binds to a CAP. An effective

15 agent can be identified by detecting an altered level of transcription of a reporter gene. For example, the level of transcription of a reporter gene due to the bridging of a DNA-binding domain and trans-activation domain by a second molecule and CAP-1 hybrids can be determined in the

20 absence and in the presence of an agent. An effective agent, which alters the association between a second molecule and a CAP, can be identified by a proportionately altered level of transcription of the reporter gene as compared to the control level of transcription in the

25 absence of the agent.

In some cases, an agent may not be able to cross the yeast cell wall and, therefore, cannot enter a yeast cell to alter the association of a CAP with a second molecule. The use of yeast spheroplasts, which are yeast

30 cells that lack a cell wall, can circumvent this problem (Smith and Corcoran, In Current Protocols in Molecular Biology (ed. Ausubel et al.; Greene Publ., NY 1989), which is incorporated herein by reference). In addition, an agent, upon entering a cell, may require "activation" by a

35 cellular mechanism, which may not be present in yeast.

Activation of an agent can include, for example, metabolic processing of the agent or a modification such as phosphorylation of the agent, which can be necessary to convert the agent into an effective agent. In this case, 5 a mammalian cell line can be used to screen a panel of agents. A transcription assay such as the yeast two hybrid system described in Example I can be adapted for use in mammalian cells using well known methods (see, for example, Fearon et al., Proc. Natl. Acad. Sci., USA 89:7958-7962 10 (1992), which is incorporated herein by reference; see, also, Sambrook et al., *supra*, 1989; Ausubel et al., *supra*, 1989).

The present invention also provides *in vitro* screening assays. Such screening assays are particularly 15 useful in that they can be automated, which allows for high through-put screening, for example, of randomly or rationally designed agents such as drugs, peptido-mimetics or peptides in order to identify those agents that effectively alter the association of a CAP with a second 20 molecule and, thereby, modulate a cell function.

An *in vitro* screening assay can utilize, for example, CAP-1 or a CAP-1 fusion protein such as a CAP-1-glutathione-S-transferase fusion protein (GST/CAP-1; see Example II). For use in the *in vitro* screening assay, the 25 CAP-1 or CAP-1 fusion protein should have an affinity for a solid substrate as well as the ability to associate with second molecule. For example, when CAP-1 is used in the assay, the solid substrate can contain a covalently attached anti-CAP-1 antibody. Alternatively, a GST/CAP 30 fusion protein can be used in the assay and the solid substrate can contain covalently attached glutathione, which is bound by the GST component of the GST/CAP-1 fusion protein. Similarly, a second molecule or a GST/second 35 molecule fusion protein can be used in an *in vitro* assay as described herein.

An *in vitro* screening assay can be performed by allowing, for example, CAP-1 or a CAP-1-fusion protein to bind to the solid support as the bait protein, then adding a second molecule and an agent to be tested.

5 Alternatively, a second molecule or a second molecule fusion protein can be used as the bait protein. Control reactions, which do not contain the agent, can be performed in parallel. Following incubation under suitable conditions, including, for example, an appropriate buffer

10 concentration and pH and time and temperature, which allow the CAP and the second molecule to bind, the amount of CAP-1 and second molecule that have associated in the absence of an agent and in the presence of an agent can be determined and compared. The association of a CAP with a

15 second molecule can be detected, for example, by attaching a detectable moiety such as a radionuclide or a fluorescent label to the second molecule and measuring the amount of label that is associated with the solid support, wherein the amount of label detected indicates the amount of

20 association of the second molecule with the CAP. By comparing the amount of specific binding in the presence of an agent as compared to the control level of binding, an effective agent, which alters the association of a CAP with a second molecule, can be identified. Such an assay is

25 particularly useful for screening a panel of agents such as a peptide library in order to detect an effective agent.

The methods for identifying an effective agent that alters the association of a CAP with a second molecule, as described above, can be used to determine

30 whether the agent can dissociate a bound second molecule/CAP-1 complex. For this purpose, a CAP is contacted with second molecule under conditions suitable for forming a second molecule/CAP complex, then the complex is contacted with the agent. The method can be performed

35 using an *in vitro* or *in vivo* assay as described above and

an effective agent can be identified by its ability to dissociate the bound complex.

The above described *in vivo* and *in vitro* assays also can be used to identify a CAP other than CAP-1. In 5 particular, the yeast two hybrid system can be performed using CD40 as the "bait" protein to screen a cDNA expression library derived from various types of CD40 expressing cells (see Example I). For *in vitro* screening, proteins encoded in the cDNA library can be expressed as 10 fusion proteins on the surface of a non-filamentous phage particle and then screened for binding to CD40 to identify a CAP other than CAP-1 (see U.S. Patent No. 5,223,409). Similar assays can be performed using a protein having a TRAF domain as the bait protein.

15 The *in vivo* and *in vitro* assays disclosed herein also can be used to identify a second molecule that binds to a CAP. For example, a second molecule such as a TRAF domain containing protein can be detected using the yeast two hybrid assay where a CAP such as CAP-1 is used as the 20 "bait" protein. A TRAF domain-containing protein can be useful for modulating the activity of a CAP in a cell and effect the function of the CAP to mediate signal transduction from CD40. The identification of a second molecule that can bind to a CAP provides an additional 25 target for modulating a cell function by contacting the cell, for example, with an effective agent that alters the association between the CAP and the second molecule.

The invention also provides a method for identifying an effective agent that alters the association 30 between CAP-1 and a second molecule in a test sample containing CAP-1 and the second molecule. As used herein, the term "test sample" means a cell or tissue specimen obtained from a subject such as a subject suspected of having a pathology characterized by an altered cell

function such as altered levels of Ig class-switching, cell proliferation or apoptosis. A test sample can be obtained, for example, during surgery or by needle biopsy. The test sample can be, for example, a soluble lysate, which is 5 obtained by treating a cell sample with a solubilizing agent such as a non-ionic detergent.

A soluble lysate or other test sample can be examined using a gel shift assay to determine whether a CAP and a second molecule are present in the cell sample and 10 associated as a bound complex. For example, the test sample can be fractionated by electrophoresis in a non-denaturing gel such as a low percentage polyacrylamide gel using a buffer containing 50 mM Tris (pH 8.5), 0.4 M glycine, 2 mM EDTA and 3% glycerol. Buffer conditions, gel 15 concentration or other parameters of electrophoresis can be optimized using well known methods such that electrophoretic separation of the free second molecule, free CAP and second molecule/CAP complex in the test sample is achieved. The identity of the free and bound molecules 20 can be determined by western blotting using antibodies specific for the second molecule or the CAP. Methods for performing western blotting, such as with enzyme or radioisotope labelled primary or secondary antibodies are well known in the art (see, for example, Harlow and lane, 25 *supra* 1988). The gel shift assay can be useful for identifying an agent that alters the association of a CAP with a second molecule by contacting a portion of a test sample with an agent and comparing the bound and free molecules in the treated sample with an untreated control 30 sample.

The invention further provides methods for identifying an agonist that can increase the level of expression of a CAP in a cell. An agonist can be introduced into a cell and, by increasing the level of 35 expression of a CAP in the cell, can modulate a function of

a cell. As used herein, "CAP agonist" means a chemical or biological molecule such as a simple or complex organic molecule, a peptide, peptido-mimetic, protein, carbohydrate or nucleotide sequence that can increase the expression or 5 activity of CAP, which can effect the level of apoptosis in a cell.

A nucleic acid molecule that encodes CAP-1 and that can be introduced into and expressed in a cell is an example of a CAP agonist. A CAP agonist also can include 10 a hormone, cytokine or other type of molecule that interacts directly or indirectly, for example, with genetic regulatory elements that control expression of a CAP in a cell. Such genetic regulatory elements include, for example, promoters, enhancers, silencers or intron 15 sequences, which can regulate protein expression at the transcriptional or translational level. For example, a CAP-1 agonist can increase the expression of CAP-1 in a cell by binding to the promoter region of the CAP-1 gene and increasing the level of transcription. A CAP-1 agonist 20 also can increase the expression of CAP-1 indirectly by binding to a regulatory protein, which, in turn, can increase transcription of the CAP-1 gene.

The invention further provides methods for modulating the level of apoptosis in a cell by contacting 25 the cell with a CAP antagonist. As used herein, "CAP antagonist" means a chemical or biological molecule such as a simple or complex organic molecule, a peptide, peptido-mimetic, protein, carbohydrate or nucleotide sequence that can decrease the expression or activity of a CAP, which can 30 modulate a function of a cell.

A CAP antagonist can be, for example, an antisense nucleotide sequence or a ribozyme, which is complementary to and can hybridize to a portion of a nucleic acid sequence encoding a CAP. Such a nucleic acid

sequence can be, for example, the CAP-1 gene or a CAP-1 mRNA transcribed from the gene. Such a CAP antagonist can be introduced into the cell and, if desired, can be expressed in the cell. The CAP antagonist can decrease the 5 expression of the CAP in a cell, which can alter the normal steady-state association of the CAP with a second molecule, thereby modulating a function of the cell.

A CAP antagonist also can include a hormone, cytokine or other molecule that interacts directly or 10 indirectly, for example, with genetic regulatory elements in a cell that result in a decreased level of expression of the CAP. Such genetic regulatory elements include, for example, a genetic promoter, enhancer, silencer or intron sequence, which can regulate protein expression at the 15 transcriptional or translational level. For example, a CAP antagonist can decrease the expression of CAP-1 in a cell by binding to the promoter region of the CAP-1 gene, thereby, decreasing the efficiency of transcription. A CAP antagonist also can decrease the expression of a CAP 20 indirectly by binding to a regulatory protein required to activate an enhancer sequence involved in transcription of the CAP gene. A CAP agonist or CAP antagonist as disclosed herein can be useful as a medicament for treating a pathology characterized by an increased or decreased cell 25 function as compared to its normal cell counterpart.

A nucleic acid sequence encoding a CAP can be expressed in a cell using well known expression vectors and gene transfer methods (see Sambrook et al., *supra*, 1989). Viral vectors that are compatible with a targeted cell are 30 particularly useful for introducing a nucleic acid encoding a CAP into a cell. For example, recombinant adenoviruses having general or tissue-specific promoters can be used to deliver a nucleic acid encoding CAP-1 into a variety of cell types in various tissues and can direct expression of 35 the nucleic acid in the target cell. Recombinant adeno-

associated viruses also are useful for introducing a nucleic acid encoding a CAP into a cell and have the added advantage that the recombinant virus can stably integrate into the chromatin of even quiescent non-proliferating 5 cells such as neurons of the central and peripheral nervous systems (Lebkowski et al., Mol. Cell. Biol. 8:3988-3996 (1988), which is incorporated herein by reference).

Such viral vectors are particularly useful where it is desirable to introduce a nucleic acid encoding a CAP 10 into a cell in a subject, for example, for gene therapy. Viruses are specialized infectious agents that can elude host defense mechanisms and can infect and propagate in specific cell types. In particular, the specificity of viral vectors for particular cell types can be utilized to 15 target predetermined cell types. Thus, the selection of a viral vector will depend, in part, on the cell type to be targeted. For example, if a neurodegenerative disease is to be treated by increasing the level of a CAP in neuronal cells affected by the disease, then a viral vector that 20 targets neuronal cells can be used. A vector derived from a herpes simplex virus is an example of a viral vector that targets neuronal cells (Battleman et al., J. Neurosci. 13:941-951 (1993), which is incorporated herein by reference). Similarly, if a disease or pathological 25 condition of the hematopoietic system is to be treated, then a viral vector that is specific for a particular blood cell or its precursor cell can be used. A vector based on a human immunodeficiency virus is an example of such a viral vector (Carroll et al., J. Cell. Biochem. 17E:241 30 (1993), which is incorporated herein by reference). In addition, a viral vector or other vector can be constructed to express a nucleic acid encoding a CAP in a tissue specific manner by incorporating a tissue-specific promoter or enhancer into the vector (Dai et al., Proc. Natl. Acad. 35 Sci., USA 89:10892-10895 (1992), which is incorporated herein by reference).

Retroviral vectors can be particularly useful for introducing a nucleic acid encoding a CAP into a cell *in vivo*. Retroviral vectors can be constructed either to function as infectious particles or to undergo only a 5 single initial round of infection. In the former case, the genome of the virus is modified so that it maintains the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. However, genes conferring oncogenic potential of these viruses is 10 destroyed. After the viral proteins are synthesized, the host cell packages the RNA into new viral particles, which can undergo further rounds of infection. The viral genome also is engineered to encode and express the desired recombinant gene.

15 In the case of non-infectious viral vectors, the helper virus genome can be mutated to destroy the viral packaging signal required to encapsulate the RNA into viral particles. However, the helper virus retains structural genes required to package a co-introduced recombinant virus 20 containing a gene of interest. Without a packaging signal, a viral particle will not contain a genome and, thus, cannot proceed through subsequent rounds of infection. Methods for constructing and using viral vectors are known in the art and reviewed, for example, in Miller and Rosman, 25 Biotechniques 7:980-990 (1992), which is incorporated herein by reference. The specific type of vector will depend upon the intended application. These vectors are well known and readily available within the art or can be constructed by one skilled in the art.

30 For gene therapy, a vector containing a nucleic acid encoding a CAP or CD40 or an antisense nucleotide sequence or ribozyme can be administered to a subject by various methods. For example, if viral vectors are used, administration can take advantage of the target specificity 35 of the vectors. In such cases, there is no need to

administer the vector locally at the diseased site. However, local administration can be a particularly effective method of administering a nucleic acid encoding a CAP or an appropriate antisense or ribozyme sequence as 5 described above. In addition, administration can be via intravenous or subcutaneous injection into the subject. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection. Injection of viral vectors into 10 the spinal fluid also can be an effective mode of administration, for example, in treating a neurodegenerative disease.

Receptor-mediated DNA delivery approaches also can be used to deliver a nucleic acid molecule encoding a 15 CAP into cells in a tissue-specific manner using a tissue-specific ligand or an antibody that is non-covalently complexed with the nucleic acid molecule via a bridging molecule (Curiel et al., Hum. Gene Ther. 3:147-154 (1992); Wu and Wu, J. Biol. Chem. 262:4429-4432 (1987), each of 20 which is incorporated herein by reference). Direct injection of a naked nucleic acid molecule or a nucleic acid molecule encapsulated, for example, in cationic liposomes also can be used for stable gene transfer into non-dividing or dividing cells *in vivo* (Ulmer et al., 25 Science 259:1745-1748 (1993), which is incorporated herein by reference). In addition, a nucleic acid molecule encoding a CAP can be transferred into a variety of tissues using the particle bombardment method (Williams et al., Proc. Natl. Acad. Sci., USA 88:2726-2730 (1991), which is 30 incorporated herein by reference). Such nucleic acid molecules can be linked to the appropriate nucleotide sequences required for transcription and translation.

A particularly useful mode of administration of a nucleic acid encoding a CAP is by direct inoculation 35 locally at the site of the disease or pathological

condition. Local administration can be advantageous because there is no dilution effect and, therefore, the likelihood that a majority of the targeted cells will be contacted with the nucleic acid molecule is increased.

5 Thus, local inoculation can alleviate the targeting requirement necessary with other forms of administration and, if desired, a vector that infects all cell types in the inoculated area can be used. If expression is desired in only a specific subset of cells within the inoculated

10 area, then a promotor, an enhancer or other expression element specific for the desired subset of cells can be linked to the nucleic acid molecule. Vectors containing such nucleic acid molecules and regulatory elements can be viral vectors, viral genomes, plasmids, phagemids and the

15 like. Transfection vehicles such as liposomes also can be used to introduce a non-viral vector into recipient cells. Such vehicles are well known in the art.

An alternative method of modulating apoptosis, proliferation or differentiation in a cell is to introduce

20 a nucleotide sequence encoding an antisense CAP, CD40 or other second molecule or a ribozyme specific for an mRNA encoding a CAP, CD40 or other second molecule into the cell. Such a nucleotide sequence can be introduced into a cell using the methods and vectors described above.

25 Chemically synthesized nucleotide sequences also can be administered directly to cells. Synthetic antisense or ribozyme oligonucleotides can be prepared using well known methods or can be purchased from commercial sources and, if desired, can incorporate nucleotide analogs to increase the

30 resistance of the oligonucleotide to degradation by nucleases in a cell. Synthetic antisense or ribozyme sequences can be active in a cell after contact with and uptake by the cell.

A nucleic acid CAP agonist or antagonist can be

35 introduced into a cell using the procedures described

above. In addition, these or other CAP agonists or antagonists of the present invention can be contacted directly with a cell *in vitro* or can be administered *in vivo* as a pharmaceutical composition containing the 5 antagonist or agonist and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such 10 as olive oil or injectable organic esters.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of a CAP-1 antagonist or agonist. Such physiologically acceptable 15 compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the 20 choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the composition. One skilled in the art would know that a pharmaceutical composition containing a CAP antagonist or agonist can be 25 administered to a subject by various routes including, for example, by direct instillation, orally or parenterally, such as intravenously, intramuscularly, subcutaneously or intraperitoneally. The composition can be administered by injection or by intubation. The pharmaceutical composition 30 also can be incorporated, if desired, into liposomes or microspheres or can be microencapsulated in other polymer matrices (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press, Boca Raton, FL 1984), which is incorporated herein by reference). Liposomes, for example, which consist of 35 phospholipids or other lipids, are nontoxic,

physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

In order to modulate apoptosis, a CAP agonist or CAP antagonist is administered in an effective amount, 5 which can be determined methods known to those in the art. As used herein, the term "effective amount" means the amount of agonist or antagonist that can have a functional effect on a cell by modulating the level of apoptosis of the cell. An effective amount of a CAP agonist or 10 antagonist administered *in vivo* is an amount that reduces symptoms associated with the disease being treated.

The total effective amount can be administered to a subject as a single dose, either as a bolus or by 15 infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which the multiple doses are administered over a more prolonged period of time. One skilled in the art would know that the concentration of CAP-1 agonist or antagonist 20 required to obtain an effective dose in a subject depends on many factors including the age and general health of the subject as well as the route of administration and the number of treatments to be administered, as well as the chemical form of the antagonist or agonist. In view of 25 these factors, the skilled artisan would adjust the particular amount so as to obtain an effective amount for subject being treated.

The present invention also provides methods for detecting the presence of a CAP such as CAP-1 in a test 30 sample by detecting the CAP protein or a nucleic acid molecule encoding the CAP. In addition, the invention provides methods for diagnosing a pathology that is characterized, in part, by an increased or decreased cell function to determine whether the abnormal cell function is 35 due, for example, to increased or decreased expression of

CAP-1 in the cell or to expression of a mutant CAP-1. The identification of such a pathology can allow for intervention therapy using an effective agent or a nucleic acid molecule or an antisense nucleotide sequence as 5 described above.

In order to diagnose a pathology that is characterized, in part, by an altered cell function, which can be due to altered expression of a CAP, a test sample can be obtained from a subject having the pathology and can 10 be compared to a control sample, which is obtained from a normal subject. The level of a CAP in the test sample can be compared to the level in the normal sample.

The level of a CAP in a cell can be determined by contacting a sample with a reagent such as an anti-CAP 15 antibody, CD40 or other second molecule, which can specifically bind a CAP. For example, the level of CAP-1 in a cell can be determined by well known immunoassay or immunohistochemical methods using an anti-CAP-1 antibody (see, for example, Reed et al., *supra*, 1992; see, also, 20 Harlow and Lane, *supra*, 1988). As used herein, the term "reagent" means a chemical or biological molecule that can specifically bind to a CAP or to CD40 or to a bound CAP/CD40 complex. For example, an anti-CAP-1 antibody or CD40 can be a reagent for CAP-1 and an anti-CD40 antibody 25 or CAP-1 can be a reagent for CD40.

Increased or decreased expression of a CAP in a cell in a test sample can be determined by comparison to an expected normal level for the CAP in a particular cell type. A normal range of particular CAP levels in various 30 cell types can be determined by sampling a statistically significant number of normal subjects. In addition, a control sample can be evaluated in parallel with a test sample in order to determine whether a pathology characterized by increased or decreased apoptosis is due to

increased or decreased expression of a CAP. The test sample can be examined using, for example, immunohistochemical methods as described above or the sample can be further processed and examined. For example,

5 an extract of a test sample can be prepared and examined to determine whether a CAP that is expressed in a cell in the sample can associate with CD40 in the same manner as a CAP from a control cell or whether, instead, a variant CAP is expressed in the cell.

10 A diagnostic assay kit incorporating a reagent such as an anti-CAP antibody, CD40 or other second molecule, which can specifically bind a CAP, can be useful for detecting a pathology due to altered CAP expression in a cell. Such a kit is particularly useful because it

15 allows for standardization of assay conditions. A kit can contain, in addition to a reagent, a reaction cocktail that provides suitable reaction conditions for performing the assay and control sample that contains a known amount of a CAP. If desired, the kit can contain an antibody that is

20 specific can specifically bind the reagent.

A diagnostic assay should include a simple method for detecting the amount of a CAP in a sample that is bound to the reagent. Detection can be performed by labelling the reagent and detecting the presence of the label using

25 well known methods (see, for example, Harlow and Lane, *supra*, 1988; chap. 9). A reagent can be labelled with various detectable moieties including a radiolabel, an enzyme, biotin or a fluorochrome. Materials for labelling the reagent can be included in the diagnostic kit or can be

30 purchased separately from a commercial source. Following contact of a labelled reagent with a test sample and, if desired, a control sample, specifically bound reagent can be identified by detecting the particular moiety.

A labelled antibody that can specifically bind the reagent also can be used to identify specific binding of an unlabelled reagent. For example, if the reagent is an anti-CAP-1 antibody, a second antibody can be used to 5 detect specific binding of the anti-CAP-1 antibody. A second antibody generally will be specific for the particular class of the first antibody. For example, if an anti-CAP-1 antibody is of the IgG class, a second antibody will be an anti-IgG antibody. Such second antibodies are 10 readily available from commercial sources. The second antibody can be labelled using a detectable moiety as described above. When a sample is labelled using a second antibody, the sample is first contacted with a first antibody, then the sample is contacted with the labelled 15 second antibody, which specifically binds to the first antibody and results in a labelled sample.

A method for determining the level of expression of a nucleic acid molecule encoding a CAP also is useful for diagnosing a pathology characterized by an abnormal 20 level of expression of a CAP. For example, a target nucleic acid molecule such as a DNA or RNA sequence encoding a CAP can be detected in a sample using a complementary nucleotide sequence. The target nucleic acid molecule can be extracted from a sample by methods well 25 known in the art (see Sambrook et al., *supra*, 1988). Methods to detect the presence of a particular nucleic acid molecule within a population of nucleic acid molecules are well known to those in the art and include, for example, Southern blotting, northern blotting, slot blotting and PCR 30 amplification (see, for example, Sambrook et al., *supra*, 1989). *In situ* hybridization also can be used to identify nucleic acids in a sample (see, for example, Pardue, in Nucleic Acid Hybridization: A practical approach (IRL Press, 1991), which is incorporated herein by reference).

To detect a nucleic acid molecule encoding a CAP in a sample, the sample is contacted with the complementary nucleotide sequence that can hybridize to a nucleic acid molecule encoding the CAP under relatively stringent 5 conditions. The nucleotide sequence can carry a detectable label, such as a radioisotope. The presence of a nucleic acid molecule encoding the CAP in the sample can be determined, for example, by detecting the level of the specifically bound nucleotide sequence. The normal level 10 of binding of the nucleotide sequence also can be determined in a control sample, which is obtained from a normal subject and contains a normal level of the CAP encoding nucleic acid molecules. An increase or a decrease in the level of nucleic acid molecules encoding a CAP in 15 the test sample compared to the normal level present in the control sample indicates a pathology characterized by an abnormal expression of the CAP. A complementary nucleotide sequence for a CAP can also be used as a primer in a PCR reaction to detect the level of nucleic acid molecules in 20 a sample encoding the CAP.

The following examples are intended to illustrate but not limit the invention.

**EXAMPLE I**

**IDENTIFICATION OF CAP-1 USING THE YEAST TWO HYBRID SYSTEM**

25 This example demonstrates the use of the yeast two hybrid system to identify cDNA sequences encoding proteins that can associate with the cytoplasmic domain of CD40.

**A. Host/Vector System**

30 The "L40 system" was used to identify cDNA sequences encoding CD40 associated proteins. *S. cerevisiae* strain L40 yeast cells were used as the host cells for the

two hybrid assay ("L40 system;" Wojtek et al., *supra*, 1993)). Strain L40 cells have a *MATA*, *trp1*, *leu2*, *his3*, *ade2*, *LYS2:(lexAop) 4-HIS3*, *URA3::(lexAop)8-lacZ* genotype and are stably transformed with histidine synthetase (*HIS3*) 5 and *lacZ* reporter genes, both of which are under the control of a *lexA* operator. Strain L40 cells were grown in YPD medium.

The "bait" protein in the yeast two hybrid system was expressed from the plasmid pBTM-116 (Vojtek et al., 10 *supra*, 1993). The bait protein, when expressed, contains a LexA DNA binding motif and, thus, associates directly with DNA acting as bait for another binding protein. The Plasmid pBTM-116 encodes the LexA DNA-binding domain under control of an *ADH* promotor (Figure 4). The presence of 15 pBTM-116 in strain L40 cells permits the cells to grow in tryptophan-deficient medium.

Plasmid pACT, also known as pSE1107, contains a promoter-controlled *GAL4* trans-activating domain (see Durfee et al., *supra*, 1993). The presence of pACT in 20 strain L40 cells permits the cells to grow in leucine-deficient medium. Physical association of the *GAL4* trans-activating domain with the DNA binding domain of LexA results in a functional transcriptional activator.

#### B. Preparation of Vectors Encoding Hybrid Proteins

25 A cDNA library derived from a human B cell was constructed in the  $\lambda$ -derived expression vector pACT as described previously (Durfee et al., *supra*, 1993). The various cDNA sequences were cloned in-frame to an upstream *GAL4* trans-activating gene sequence.

30 The cDNA encoding two cytosolic fragments of CD40 (positions 216-277 and positions 225-269) were generated by PCR from the plasmid pCDM8-CD40 (Stamenkovic et al., EMBO

J. 8:1403-1410 (1989), which is incorporated herein by reference) using the following primers containing Eco RI (underlined) and Bcl I (italic) sites (bold indicates stop codon) : CD40 (positions 216 - 277),  
5 5'-GGAATTCAAAAAGGTGGCCAAG-3' (forward primer; SEQ ID NO: 3) and 5'-*TGATCAT*CACTGTCTCCTGCAC-3' (reverse primer; SEQ ID NO: 4); and CD40 (positions 225-269),  
10 5'-GGAATTCAAGGCC~~CCCC~~ACCCCAAG-3' (forward primer; SEQ ID NO: 5) and 5'-*TGATCA*ACTCTTTGCCATCCTC-3' (reverse primer; SEQ ID NO: 6). The PCR products were digested with Eco RI and Bcl I, then directly cloned into the Eco RI and Bam HI sites of the yeast two hybrid plasmid pBTM-116 in-frame with the upstream LexA DNA-binding domain sequences (Vojtek et al., *supra*, 1993).

15 For expression of LexA-Fas, a cDNA sequence encoding a cytoplasmic domain fragment of Fas (positions 191-335; Itoh et al., *supra*, 1991) was generated by PCR (Higuchi et al., *supra*, 1990) from plasmid pBSAP014.1 (Oehn et al., *J. Biol. Chem.* 267: 10709-10715 (1992) and Sato et 20 al., *Proc. Natl. Acad. Sci. (USA)* 91:9238-9242 (1994), both of which is incorporated herein by reference) using the following primers containing Eco RI (underlined) and Bcl I (italics) sites (bold indicates STOP codon, TCA), as follows: 1) 5'-GGAATTCAAGAGAAAGGAAGTACAG-3' (forward 25 primer; SEQ ID NO: 7); 2) 5'-*GTGATCA*CTAGACCAAGCTTGAT (reverse primer; SEQ ID NO: 8). The PCR product was digested with Eco RI and Bcl I and cloned into EcoRI/BamHI digested pBTM-116 to produce pBTM/CD40 (positions 191-335).

30 For expression of a LexA-TNF-R2 fusion protein, the cytosolic domain of TNF-R2 (positions 228-461) was amplified by PCR from a p-Bluescript-p80-TNF-R2 plasmid (Smith et al., *Science* 248:1019-1023 (1990), which is incorporated herein by reference) using the following primers containing Eco RI (underlined) and Sal I (italics) 35 sites (bold indicates a stop codon, TTA)

5'-GGAATTCAAAAGAAGCCCTTGTGCCT-3' (forward primer; SEQ ID NO: 9) and 5'-GGTCGACTTAACTGGGCTTCATCCCA-3' (reverse primer; SEQ ID NO: 10). The PCR product was digested with Eco RI and Sal I and cloned into EcoRI/XhoI-digested pBTM-116 to 5 produce pBTM/TNF-R2 (positions 228-461).

Additional pBTM-116 plasmids encoding portions of the Bcl-2 (positions 83-218), Ha-Ras (V12), and Lamin-C proteins were produced as described previously (Sato et al., *supra*, 1994; Vojtek et al. *supra*, 1993).

10

#### C. Assay Methods

Plasmid DNA was transformed into yeast cells by the LiCl method (Gietz et al., *Nucl. Acids Res.* 20:1425 (1992); Schiestl et al., *Curr. Genet.* 16:339-346 (1989), 15 each of which is incorporated herein by reference). 0.5 mg of pACT-cDNA library DNA mixed with 1 mg of denatured salmon sperm carrier DNA was used for transformation of ~5 X 10<sup>9</sup> L40-strain cells. Transformed cells were grown in complete minimal medium lacking tryptophan or leucine as 20 necessary to select for the presence of pBTM or pACT derived plasmids, respectively. In addition, growth in histidine-deficient medium indicated formation of transcriptionally active LexA/GAL4 complex.

#### D. Selection of CAP Candidates:

25 The pBTM/CD40 (positions 216-277) plasmid was introduced into L40 cells, which contain histidine synthetase (HIS3) and  $\beta$ -galactosidase (lacZ;  $\beta$ -gal) reporter genes under the control of lexA operators. The resulting transformants were selected for ability to grow 30 on medium lacking tryptophan, since the pBTM-116 plasmid contains a TRP1 gene that complements the defect in the host strain. L40 cells containing the pBTM/CD40 (positions 216-277) then were transformed with the pACT-B cell cDNA

library. Transformants containing both pBTM and pACT plasmids were selected by growth on media lacking both leucine and tryptophan.

Colonies expressing a cDNA sequence encoding a candidate CAP were identified initially by their ability to grow in medium lacking histidine due to expression of the lexAop/HIS3 reporter gene construct in the yeast cells. Approximately 31 million transformants were screened and 2,640 His<sup>+</sup> colonies were identified. These 2,640 clones were examined further using a  $\beta$ -gal colorimetric filter assay as described previously (Sato et al., *supra*, 1994) to identify expression of the lacZ reporter gene, which is under the control of a lexA operator. Following this second screening procedure, 166 positive clones were obtained.

The 166 clones were cured of LexA/CD40-encoding plasmids by growing the cells in tryptophan-containing medium. The cured cells then were mated against a panel of a-type yeast (strain NA-8711-A (*a, leu2, his3, trp1, pho3, pho5*)) plasmids containing one of the following LexA fusion proteins: cytosolic domain of CD40 (positions 216-277), CD40 (positions 225-269), cytoplasmic domain of Fas (positions 191-335), Bcl-2 (positions 83-218) Ras V12 and Lamin C. The mated cells were selected for growth in medium lacking tryptophan and leucine to obtain cells that contained both a pACT-B cell clone and a LexA-fusion protein. Colonies were transferred to histidine-deficient plates or histidine-containing media with X-Gal. Colonies expressing  $\beta$ -gal activity were identified using a plate assay and a filter assay. The plate assay was performed on the X-Gal containing plates ~12 hr after plating according to previous methods (see Chien et al., *Proc. Natl. Acad. Sci., USA* 88:9578-9582 (1991), which is incorporated herein by reference). The  $\beta$ -gal filter assay was performed after 1 hr culture as described previously (Sato et al., *supra*,

1994; Breeden and Nasmyth, Cold Spring Harbor Symp. Quant. biol. 50:643-650 (1985), which is incorporated herein by reference).

Of the 166 clones tested, two clones were found  
 5 (pACT121 and pACT2229) that reacted with the LexA/CD40 protein but not with the other fusion proteins (see Table 1). DNA sequence analysis showed that the two clones contained identical cDNA inserts fused to the upstream GAL4 trans-activating domain, suggesting that the two clones  
 10 arose as library-amplified copies of a single cDNA. The sequence of the cDNA inserts of the two clones showed an open reading frame of 543 base pairs (bp; 181 amino acids) followed by a stop codon and 3' untranslated region of ~457 bp. The cDNA was named CAP-1 for CD40 associated  
 15 protein-1.

TABLE I  
INTERACTION OF CAP-1 (pACT2229/GAL4 FUSION) WITH VARIOUS LexA FUSION PROTEINS

20	pBMT-116 (LexA FUSION)	HIS <sup>-</sup>	Growth	β-GAL
	CD40 (216-277)	+	+	
	CD40 (225-269)	+	+	
	TNFR2 (288-461)	-	-	
25	Fas (191-335)	-	-	
	Ras(V12)	-	-	
	Bcl-2 (83-218)	-	-	
	Lamin-C	-	-	

**EXAMPLE II**  
**IN VITRO ASSAYS TO IDENTIFY A CAP**

This example describes methods for constructing fusion proteins and producing labelled proteins useful  
5 for detecting and characterizing a CAP *in vitro*.

**A. Production of GST Fusion Proteins**

GST-fusion proteins were cloned into pGEX plasmids (Pharmacia, Piscataway, NJ) and expressed in *E. coli*. pBTM 116-CD40 (positions 225-269), Fas and TNF-R2  
10 plasmids were digested with Eco RI and either Xho I or Sal I and the inserts subcloned in-frame with the upstream GST sequences in pGEX-4T-1. For GST-CD40 (positions 220-277), a Bal I fragment encoding amino acids 220-277 was excised from pCDM8-CD40 and subcloned  
15 initially into the Hinc II site of Bluescript pSKII (Stratagene, Inc.; La Jolla CA). The pSKII-CD40/BalI plasmid was then cut with XhoI and EcoRI and the fragments containing CD40 sequences were each subcloned into a modified version of pGEX-2T-1 in-frame with the  
20 GST sequence. For GST-TNF-R1, the cytoplasmic domain of TNF-R1 was PCR amplified from the plasmid pUC19-p55-TNF-R1 (Loetscher et al., Cell 61:351-359 (1990), which is incorporated herein by reference) using the following primers 5'-GGGATCCGCTACCAACGGTGGAAG-3' (forward primer; 25 SEQ ID NO: 11) and 5'-GGTCGACTCATCTGAGAAGACTGGG-3' (reverse primer; SEQ ID NO: 12), digested with Bam HI and Sal I and subcloned into pGEX-5X-1. For GST-CAP-1, the pACT2229 cDNA was digested with Bgl II, and the CAP-1 cDNA (positions 363-543) was subcloned into pGEX-3X.  
30 GST-fusion proteins were produced in either DH5- $\alpha$ -F' or HB101 cells transformed with pGEX plasmids. Overnight cultures were diluted 1:10 into LB medium containing 50  $\mu$ g/ml ampicillin to make 0.5 liter cultures

and were incubated at 30°C and 250 revolutions per minute (rpm) for an additional 2 hr, after which isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) was added (1 mM) and the cultures were incubated for 8 to 16 hr further at 30 °C 5 and 250 rpm. Cells were recovered by centrifugation, resuspended in ~10 ml of phosphate buffered saline (PBS) pH 7.4 (12.5 mM NaPO<sub>4</sub> and 0.15 M NaCl) containing 1% Triton-X100, 1 mM PMSF, and 1 mg/ml lysozyme and sonicated on ice using 5 bursts of 30 seconds each from a 10 1.6 mm tip (Ultrasonicator Model XL-2020; Heat Systems, Inc., Farmingdale NY). After centrifugation at 8,000 x g for 10 min, the resulting supernatant was mixed with 1 ml of glutathione-Sepharose 4B (Pharmacia, Inc.) for 1 hr at 4°C. The beads were washed 2X in PBS containing 1% 15 Triton-X100 and the amount of bound GST protein was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie-blue staining of a sample of the protein released by boiling an aliquot of the beads in SDS sample buffer (see Harlow and lane, 20 *supra*, 1988).

#### B. Characterization of CAP binding to CD40

The disclosed methods demonstrate *in vitro* binding between CAP-1 and CD40.

##### 1. Labelling of CAP-1

25 The CAP-1 cDNA was PCR amplified using a forward primer containing a T7 promoter (5'-TAATACGACTCACTATAGGGAGACCACATGGATGATGTATAACTATC ATTTC-3'; SEQ ID NO: 13) and a reverse primer (5'-CTACCAGAATTGGCATGCCGGTAGAGGTGTGGTCA-3'; SEQ ID NO: 30 14) from 1  $\mu$ g pACT2229 plasmid DNA. One-tenth of the resulting PCR product (approximately 0.1  $\mu$ g) was then directly transcribed and translated *in vitro* in the

presence of  $^{35}\text{S}$ -methionine using 12.5  $\mu\text{l}$  of reticulocyte lysate (TNT-Lysate®; Promega, Inc.; Madison, WI).

### 2. Binding of Labelled CAP-1 to GST Fusion Proteins

Glutathione-Sepharose beads containing 5  $\mu\text{g}$  of

- 5 GST fusion proteins (i.e., bait proteins) were incubated with 10  $\mu\text{l}$  *in vitro*-translated  $^{35}\text{S}$ -CAP-1 protein for 16 hr at 4°C in a buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM DTT, 2 mM EDTA, 0.1 % NP-40, 1 mM PMSF and 1  $\mu\text{g}/\text{ml}$  leupeptin. The beads were washed vigorously 5x in
- 10 the above buffer, pelleted by centrifugation, boiled in SDS-PAGE sample buffer and analyzed by SDS-PAGE and fluorography (Harlow and Lane, *supra*, 1988). The binding results (not shown) indicated that CAP-1 interacted specifically with GST-fusion proteins containing the
- 15 nearly full-length CD40 cytoplasmic domain (positions 220-277) as well as the conserved subregion of the CD40 cytosolic domain (positions 225-269). No interactions were detected between CAP-1 and GST-fusion protein containing the cytosolic domains of Fas, TNF-R1, or
- 20 TNF-R2 as well as GST itself. Interestingly, CAP-1 did interact with GST-CAP-1, implying that CAP-1 can homodimerize. The specificity of these results was further confirmed by experiments where a variety of other  $^{35}\text{S}$ -labeled *in vitro*-translated proteins, including Bcl-2,
- 25 Bax, Lyn were found not to bind to the above GST-CD40 fusion proteins under the same conditions used for binding to  $^{35}\text{S}$ -CAP-1 (data not shown).

### 3. Binding of GST-CAP-1 to CD40 from a Cell Lysate

For experiments with cell lysates,  $2 \times 10^7$

- 30 RS11846 B-cell lymphoma cells were lysed in 0.25 ml of ice-cold Tris buffered saline (TBS) pH 7.4 (10 mM Tris and 150 mM NaCl) including 5 mM EDTA, 1% Triton-X100, with protease inhibitors PMSF, aprotinin, leupeptin,

benzamidine, and pepstatin. Nuclei and cellular debris were removed from the cell lysate by centrifugation as described (Reed et al., Canc. Res. 51:6529-6538 (1991)). The resulting supernatants were then incubated with 5 either 5  $\mu$ g of affinity-purified GST-CAP-1 (positions 363-543) or GST as a control protein (bait proteins) immobilized on 50  $\mu$ l of glutathione-Sepharose beads for ~16 hr at 4°C, then washed 10x in 50 mM Tris pH 7.4 containing 150 mM NaCl, 5 mM EDTA, 0.1 % NP-40, 1 mM 10 PMSF, 1  $\mu$ g/ml leupeptin. Proteins adsorbed to beads were eluted by boiling in SDS-PAGE sample buffer, electrophoresed in SDS-PAGE gels containing 12% acrylamide and then transferred to nitrocellulose for western blotting. The resulting blots were preblocked in 15 TBS buffer containing 5% non-fat milk and 3% bovine serum albumin and then incubated with 2  $\mu$ l/ml of anti-human CD40 monoclonal antibody B-B20 (Biosource, Inc., Camarillo CA) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (see Reed et al., *supra*, 20 1992; Hanada et al., Canc. Res. 53:4978-4986 (1993), which is incorporated herein by reference). The presence of bound peroxidase was detected using an emission chemiluminescence method performed according to the manufacturer's instructions (Amersham, Inc., Arlington 25 Heights IL).

Western blotting with the anti-CD40 antibody identified a band in the lymphoma cell lysate of about 50 kda indicating the presence of CD40 in the lysate. A similar size band was seen in the lane where the lysate 30 was reacted with the GST-CAP-1 fusion protein but not in the lane where the GST-control protein was reacted with the lysate, indicating that the GST-CAP-1 can bind specifically to CD40 in the cell lysate.

C. Screening a cDNA expression library

A cDNA library can be screened to identify a new CAP or to identify clones providing further cDNA sequence to complete the open reading frame (ORF) of the 5 partial cDNA clone that was used to screen the library.

To identify a new CAP, a  $^{32}\text{P}$ -labeled GST/CD40 protein can be used for direct screening of a  $\lambda$ gt11 cDNA expression library, which can be obtained from a commercial source (Clontech; Palo Alto CA) or constructed 10 using well known methods (Sambrook et al., *supra*, 1989). Radiolabeled GST control protein or GST/CD40 is obtained as described above. Phage containing the library clone sequences are transferred to nitrocellulose filters, which are placed in a plastic bag. Hybridization buffer 15 (1% milk, 20 mM Hepes-KOH, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl<sub>2</sub>) is added and approximately 250,000 cpm/ml GST/CD40 (or GST) are added. Filters are incubated at 4 °C, overnight, in the presence of a 20-fold molar excess of unlabeled GST protein (see, for 20 example, Kaelin et al., *supra*, 1992).

Positive plaques are selected and subjected to further rounds of screening as above. After 3-4 rounds of screening, the cDNA inserts of plaque purified phages are amplified by PCR using primers that flank the vector 25 cloning site or, alternatively, are liberated from the phage by restriction digestion of purified phage DNA. These cDNA sequences are subcloned into the EcoRI site of the pET vector, pET5c (Novagen; Madison WI), which produces T7/Protein 10 fusion proteins that are in-frame 30 with the inserted cDNA. Expression of the recombinant proteins is induced in *E. coli* using IPTG, fractionated by SDS-PAGE and transferred to nitrocellulose filters. The resulting blots are hybridized to the same GST/CD40 fusion protein from the original screening using the

hybridization method described above for phage screening. Clones that produce CAP's, which bind the GST/CD40 probe, are sequenced and their amino-acid sequences are deduced. These sequences are checked against nucleotide and 5 protein databases to identify novel or previously described proteins.

New CAP's obtained as described above or CAP's selected from the yeast two hybrid assay are used to produce hybridization probes to screen various tissues by 10 northern blot analysis. Appropriate clones can then be used as hybridization probes to screen commercially-available lambda phage cDNA libraries prepared from an appropriate tissue to obtain a cDNA encoding the entire ORF or to obtain overlapping cDNA sequences which 15 together encode the entire ORF of the various CAP's. In a specific example, the cDNA insert of CAP-1 clone pACT2229 clone was excised with XhoI, <sup>32</sup>P-labeled by a random primer method (see Sambrook et al. *supra*, 1989), and used to screen a human fetal brain cDNA library in 20 λ-gt11 (Clontech, Inc.; Palo Alto CA). Two cDNA clones were obtained having insert sizes of ~2.0 kba (kilobase pair) and 0.1 kba. DNA from the new clones were PCR amplified using primers flanking the cloning site in λ-gt11, and the resulting PCR products were subcloned 25 into a Eco RV-digested, T-tailed pSKII plasmid and their DNA sequence determined by the dideoxynucleotide method. The DNA sequence of the longer of these two clones (pSK-7) extended the coding region of the original cDNAs in the 5'-direction by another 282 amino-acids but the lack 30 of a consensus translation initiation site suggested that the CAP-1 ORF was still incomplete.

A 5'- or 3'-RACE method is useful to obtain cDNA sequence data to complete a full ORF of a partial cDNA clone. The missing 5' ORF of the CAP-1 sequence was 35 obtained using a 5' RACE method to directly amplify the

regions of the cDNA from reverse transcribed RNA (Frohman et al., Proc. Natl. Acad. Sci., USA 85:8998-9002 (1988), which is incorporated herein by reference). For 5' RACE, 10 µg of total RNA from Raji B-cell lymphoma cells was 5 reverse-transcribed using a specific primer 5'-GCGTTAACTGCTCTGCACAA-3' (SEQ ID NO: 15) and recombinant Moloney murine leukemia virus reverse transcriptase (Gibco/BRL, Inc.; Gaithersburg MD). The resulting cDNA was subjected to homopolymeric tailing 10 using dCTP and terminal deoxynucleotidyl transferase using a kit from BRL/GIBCO, and then PCR amplified using a universal 5'-forward primer provided by the manufacturer and a 3'-CAP-1-specific reverse primer 5'-GTACATTTGGACTTGAAGA-3' (SEQ ID NO: 16). The final 15 PCR product was subcloned into T-tailed pSK-II and a clone (pSK-5') was obtained and the DNA insert sequenced. The insert of pSK-5' provided an additional ~ 378 bp of DNA sequence to complete the ORF of CAP-1 (see Example III for the complete cDNA sequence of CAP-1).

20

**EXAMPLE III**  
CHARACTERIZATION OF CAP-1

This example describes the characteristics of human CAP-1 .

The complete ORF of CAP-1 was obtained by 25 combining the DNA sequence insert present in the original pACT2229 clone insert with the sequence inserts from clones pSK-7 and pSK-5' (see Example I and II for details). The complete CAP-1 ORF encodes a 543 amino-acid protein, having an estimated molecular mass of 30 ~62 kDa (Figure 1). The full length sequence is based on a predicted translation initiation site that conforms in 6 of 7 positions to the Kozak consensus sequence (Kozak, J. Biol. Chem. 266:19867-19870 (1991)). Stop codons were found in all three reading-frames upstream of the longest

ORF (nucleotide sequence submitted to Genbank). The absence of a polyadenylation sequence in the 3' untranslated region of the CAP-1 cDNAs (pACT2229 and pSK-7) suggests that the complete 3' end of the CAP-1 cDNA 5 sequence remains to be determined.

A search of the CAP-1 nucleotide sequence against the available databases using the BLAST program 9 (Pearson and Miller, Meth. Enzymol. 210: 675-601 (1992), which is incorporated herein by reference) revealed 26% 10 and 30% overall amino-acid sequence homology with TRAF1 and TRAF2, two putative signal transducing proteins that have recently been shown to bind to the cytosolic domain of TNF-R2 (Rothe et al., *supra*, 1994). The strongest homology was located in the C-terminal regions of these 15 proteins, corresponding to the TRAF domains of TRAF1 and TRAF2 (not shown). The cytosolic domain of CAP-1 is located between residues 384 to 540, and has 57% and 59% amino-acid identity to the analogous domains in TRAF1 and TRAF2, respectively. TRAF1, TRAF2 and CAP-1 are examples 20 of second molecules that contain a TRAF domain and can bind to CAP-1. Due to this high degree of homology, the cytosolic domain of CAP-1 is referred to as a TRAF domain.

Like TRAF2, the CAP-1 protein contains a RING 25 finger domain consensus sequence near its NH<sub>2</sub>-terminus (residues 53-91). This sequence is believed to form a structure that binds two zinc atoms and is found in several regulatory proteins that can bind to either DNA or RNA, including the DNA repair genes RAD18 of *S. cerevisiae* and UVS-2 of *Neurospora crassa*, the human 30 SS-A/Ro ribonucleoprotein, the Bmi-1, c-dbl, Pml, and RET proto-oncogenes, the recombinase RAG-1, and the human RING-1 protein (reviewed by Freemont, *supra*, 1993; Schwabe and Klug, *supra*, 1994; Barlow et al., J. Mol.

Biol. 237:201-211 (1994)). The RING finger domains of CAP-1 and TRAF2 are 42% identical.

Following the RING finger motif of CAP-1 are three zinc finger-like domains. Two of the domains (ZF2 and ZF3) contain a consensus sequence, which is found in several DNA-binding proteins including the *Xenopus* transcription factor TFIIIA (Berg, J. Biol. Chem. 265:6513-6516 (1990) as well as some RING-finger proteins such as RAD18, and UVS-2 (reviewed by Freemont). The 5 third zinc-finger-like domain (ZF1) is found in CAP-1 (between residues 117-141), but the spacing between the first two cysteines is longer than usual in this case (6 instead of the usual 2 to 4 residues). The TRAF2 protein contains four zinc finger motifs downstream of its RING 10 15 finger domain. The absence of RING and zinc finger domains in TRAF1 indicates it is more distantly related to the other TRAF family members CAP-1 and TRAF2.

Comparison of the TRAF domain of CAP-1 with those of TRAF1 and TRAF2 suggests that the essential 20 conserved region between these domains can be narrowed to a region of ~150 amino acid residues representing position 385-536 of CAP-1, 254-406 of TRAF1 and 346-497 of TRAF2. The TRAF 1 and TRAF2 proteins exhibit additional homology that extends further in the N- 25 terminal direction to include residues 181-406 of TRAF1 and 272-497 of TRAF2 (Rothe et al., *supra*, 1994). Taken together, these data indicate that CAP-1 is a novel 30 homolog of TRAF2 and a new member of the family of putative signal transducing proteins that contain TRAF-domains.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, 5 the invention is limited only by the claims.